UNIVERSITI PUTRA MALAYSIA

A STRUCTURAL STUDY ON THE SPECIFICITY OF F1 PROTEASE

AZIRA MUHAMAD.

FBSB 2005 34

A STRUCTURAL STUDY ON THE SPECIFICITY OF F1 PROTEASE

By

AZIRA MUHAMAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

December 2005

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

A STRUCTURAL STUDY ON THE SPECIFICITY OF F1 PROTEASE

By

AZIRA MUHAMAD

December 2005

Chairman : Assoc. Professor Raja Noor Zaliha Raja Abd. Rahman, PhD

Faculty : Biotechnology and Biomolecular Sciences

Specificity studies of a thermostable alkaline serine protease F1 with its substrates were carried out through computational docking method. Structures of a series of synthetic peptide substrates were docked to the active site of the homology modelled F1 protease using AutoDock 3.0.5. The resulting clusters of the substrates that were docked were analysed by inspecting the energetic results and the orientation of each cluster to determine the arrangement of productive binding. The amino acids of the binding site that participated in the hydrophobic and hydrogen-bond interactions were also determined. Docking results showed that all substrates tested bound near the catalytic residues with SucAAPFpNA, the biggest substrate, showing the most negative docked energy value (E_{docked} = -18.75 kcal/mol). Smaller substrates such as GpNA and AApNA showed higher docked energy (E_{docked} = -7.77 kcal/mol and -8.77 kcal/mol, respectively). The

best docked structure of each substrate was determined from the clusters. It was found that most of the lowest E_{docked} conformations display the best docked orientations with respect to the least distance calculated between the carbonyl carbon of the substrate P1 residue and γ-oxygen of the Ser226 catalytic triad. From the results, it also demonstrated that S1, S2 and S4 subsites of the enzyme play a critical role in determining the substrate specificity of F1 protease from the point of view that bigger-sized substrates such as SucAAPFpNA and SucAAPLpNA showed more favourable E_{docked}. This work also support the hypothesis that the catalytic serine and histidine residues were essential in catalysis as well as in stabilizing the enzyme-substrate complex for binding.

Validation of computational study was carried out through biochemical assay. It was found that SucAAPFpNA was the most preferred substrate for the enzyme with specific activity of 3.079 U/mg followed by SucAAPLpNA at 1.016 U/mg. SucAAPFpNA was also observed to show the highest binding affinity towards the protease (K_m = 1.26mM) and the highest catalytic ratio (1.226 $min^{-1}.mM^{-1}$) compared to the other substrates tested. Similar to computational observations, smaller peptides showed lower specific activity and binding affinity towards the protease. Rank-order of the substrates tested for the docking and experimental methods were found to be similar for the top two substrates, with lesser agreement for the other substrates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

KAJIAN STRUKTUR SPESIFIKASI F1 PROTEASE

Oleh

AZIRA MUHAMAD

Disember 2005

Pengerusi : Profesor Madya Raja Noor Zaliha Raja Abd. Rahman, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Suatu penyelidikan spesifikasi terhadap serine protease F1 yang stabil suhu tinggi dan bersifat alkali bersama substrat telah dijalankan melalui kaedah percamtuman pengkomputeran. Struktur-struktur peptida sintetik telah dicantum ke tapak aktif F1 protease yang telah dimodelkan secara homologi dengan menggunakan AutoDock 3.0.5. Kelompok yang telah dihasilkan oleh substrat-substrat yang telah dicantum, telah dianalisa keputusan tenaga dan orientasi setiap kelompok untuk menentukan susunan cantuman yang produktif. Asid amino di tapak pengikat yang menyertai interaksi hidrofobik dan ikatan hidrogen juga telah ditentukan. Percantuman substrat-substrat pada enzim ini menunjukkan yang kesemua substrat yang telah diuji mengikat berhampiran residu katalitik di mana SucAAPFpNA, iaitu substrat yang paling besar, menunjukkan nilai E_{docked} yang paling rendah, -18.75 kcal/mol. Substrat-substrat yang lebih kecil seperti GpNA dan AApNA menunjukkan nilai E_{docked} yang lebih tinggi, masing-masing pada -7.77

kcal/mol dan -8.77kcal/mol. Struktur yang paling sesuai telah ditentukan daripada setiap kelompok. Didapati bahawa kebanyakkan konformasi E_{docked} yang paling rendah mempunyai orientasi yang paling sesuai berpunca dari jarak di antara karbon karbonil substrat residu P1 dan γ-oksigen Ser226 triad katalitik yang paling rendah. Cantuman substrat-substrat itu juga menunujukkan subtapak S1, S2 dan S4 enzim memainkan peranan yang kritikal dalam penentuan spesifikasi substrat bagi F1 protease kerana didapati bahawa subtrat-substrat yang lebih besar seperti SucAAPFpNA dan SucAAPLpNA menunjukkan nilai E_{docked} yang lebih rendah. Kajian ini mengesahkan bahawa residu-residu katalitik seperti serine dan histidine adalah penting dalam katalisis dan dalam pengstabilan kompleks enzim-substrat untuk percantuman.

Kajian kesahihan telah dijalankan dengan melakukan asai biokimia. Didapati bahawa SucAAPFpNA adalah substrat yang paling digemari oleh enzim ini dengan aktiviti spesifik, 3.079 U/mg, diikuti SucAAPLpNA pada 1.016 U/mg. SucAAPFpNA juga didapati mempunyai afiniti pengikatan yang paling tinggi terhadap protease (K_m = 1.26mM) dan ratio katalitik paling tinggi (1.226 $min^{-1}.mM^{-1}$) berbanding dengan substrat-substrat lain yang diuji. Peptida yang lebih kecil didapati mempunyai spesifik aktiviti dan afiniti pengikatan yang rendah terhadap F1 protease, seperti yang telah diperolehi dari analisis pengkomputeran. Persamaan yang didapati daripada urutan substrat-substrat yang diperolehi daripada percantuman pengkomputeran

dan kaedah eksperimental adalah pada tempat kedua teratas, substrat-substrat lain kurang menunjukkan kaitan.

ACKNOWLEDGMENT

I wish to express my deepest gratitude to my supervisor, Assoc. Prof. Dr. Raja Noor Zaliha Raja Abd. Rahman for her constant guidance and encouragement throughout the duration of this study. My sincere appreciation is extended to my co-supervisors, Prof. Dr. Abu Bakar Salleh, Prof. Dr. Mahiran Basri and Dr. Habibah A. Wahab. Their critical comments and support have been an invaluable source of motivation. My gratitude is also extended to Assoc. Prof. Dr. Mohd. Basyaruddin Abd. Rahman for his helpful comments and guidance.

I would like to thank all my lab mates, Ferrol, Kak Ain, Kak Ina, Kak Naz, Leow, Ghanie, Rofandi, Kak Lia, Kok Whye, Brother Mohd, Aiman, Sha, Shuk, Sue, Yob, Ada, Chee Fah and Tengku for their friendship and support during my stay at the lab. You guys have been really helpful. My sincere appreciation also goes especially to Bimo for his invaluable encouragement and comments. You have been a real source of inspiration. Thank you for being so patient and thank you so much for the long hours you've spent for me.

Last but not least, my heartfelt gratitude goes to my family, Bapak, Mak, Abang, Kak Yani and Alen. Thank you for your patience, encouragement and support.

I certify that an Examination Committee met on 14th December 2005 to conduct the final examination of Azira Muhamad on her Master of Science thesis entitled "A Structural Study of the Specificity of F1 Protease from *Bacillus stearothermophilus*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Mohd Arif Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Suhaimi Napis, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Nor Aripin Shamaan, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sharifuddin M Zain, PhD
Associate Professor
Faculty of Science
Universiti Malaya
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 JAN 2006

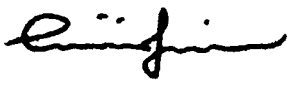
This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

RAJA NOOR ZALIHA RAJA ABD. RAHMAN, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

ABU BAKAR SALLEH, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

MAHIRAN BASRI, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

HABIBAH A. WAHAB, PhD
Lecturer
School of Pharmaceutical Sciences
Universiti Sains Malaysia
(Member)

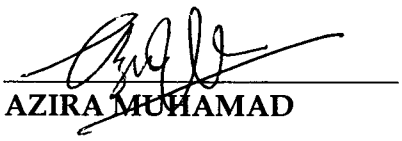


AINI IDERIS, PhD
Professor/Dean
Schoolof Graduate Studies
Universiti Putra Malaysia

Date: 07 FEB 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



AZIRA MUHAMAD

Date: 30.12.2005

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$\Delta G_{binding}$	Free energy of binding
Å	Angstrom
dH_2O	distilled water
E_{docked}	Docked energy
h	hour
HF	Hartree-Fock
K	Kelvin
kcal/mol	kilocalorie per mol
M	Molar
mg	milligram
ml	milliliter
mM	milliMolar
min	minute
RESP	restrained electrostatic potential
RMSd	root mean square deviation (s)
µg	microgram
µl	microliter
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Proteases are enzymes that cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus. There are four mechanistic classes of proteases, they are serine proteases, cysteine proteases, aspartic proteases and metallo proteases. Two distinct families can be observed for serine proteases, which are the chymotrypsin family and the subtilisin family which include the bacterial enzymes such as subtilisin (Rao *et al.*, 1998). An example of subtilisin serine protease is *Bacillus stearothermophilus* protease F1. *B. stearothermophilus* was isolated from decomposed oil palm branches and its protease showing the characteristics of an extremely thermostable alkaline protease. *B. stearothermophilus* was shown to have a high degree of thermostability, able to grow up to 80°C with a pH range from 8.0 to 10.0. It has an optimum growth rate at 70°C and pH 9 (Rahman *et al.*, 1994).

Proteases differ in their properties such as substrate specificity, active site and catalytic mechanism. Structure-based mutational analysis of serine proteases specificity has produced a large database of information useful in addressing biological function and in establishing a basis for targeted design efforts (Perona and Craik, 1995). Despite extensive research on proteases, relatively little is known about the factors that control their

specificities. These questions that are difficult to answer experimentally, might be resolved theoretically.

Molecular modelling methods have been used in many ways to address problems in structural biology. The overall aim of modelling methods will often be to try to relate biological activity to structure (Forster, 2002). Molecular modelling is a general term that covers a wide range of molecular graphics and computational chemistry techniques used to display, build, manipulate, simulate and analyze molecular structure and to calculate properties of these structures. Through modelling, the understanding of properties, three-dimensional conformations, and enzymatic mechanism can also be improved.

One of the most useful areas of application of molecular modelling is the approach of fitting together, or docking, a protein to a second molecule. Molecular docking is important in understanding possible interactions between a protein and a ligand in the formation of a biologically important protein-ligand complex. It is also a suitable tool to estimate interaction energy of the binding step by means of rigid energy minimization methods (Teodoro *et al.*, 2001).

There are many docking software available and most of these programs are free for public. One of the widely used programs is AutoDock.

AutoDock is an automated docking program of flexible ligands to receptors. It was developed by Olson's group in 1990 (Goodsell and Olson, 1990). AutoDock uses free energy of the docking molecules using three-dimension potential-grids. The search algorithms implemented include Simulated Annealing (SA), Genetic Algorithm (GA) and Lamarckian GA (LGA) with GA + local search (LS) hybrid. The search and score method in AutoDock involves exploration of the configuration spaces available for interaction between ligand and receptor and it evaluates and ranks configurations using a scoring system which is the binding energy (Morris *et al.*, 1998).

In order to further understand the molecular basis of substrate specificity of *B. stearothermophilus* F1 protease, the following objectives were implemented in view of this research:

1. To find the best substrate based on binding energies obtained from docking procedure
2. To study the interaction of F1 protease with its substrates
3. To validate results obtained through computational studies by experimental methods

CHAPTER 2

LITERATURE REVIEW

2.1 Proteases

Proteases comprise two groups of enzymes, the endopeptidases and the exopeptidases. The exopeptidases act only near the ends of polypeptide chains at the N or C terminus while the endopeptidases act preferentially in the inner regions of peptide chains away from the N and C termini. These proteases are classified according to their catalytic mechanisms. There are four mechanistic classes recognized by International Union of Biochemistry and Molecular Biology. They are serine proteases, cysteine proteases, aspartic proteases and metallo proteases (Rao *et al.*, 1998).

Serine proteases have been grouped into six distinct families of which the chymotrypsin-like clan and the subtilisin-like clan are the two largest family (Siezen and Leunissen, 1997). They have the same active site geometry and the catalysis proceeds via the same mechanism but the general three-dimensional structure is different in the two families. Three residues (histidine, aspartic acid and serine) that form the catalytic triad are essential in the catalytic process of the enzyme.

Plant proteases such as papain and bromelain are included in the cysteine proteases family. The essential cysteine and histidine residues play the same role as serine and histidine residues in serine proteases during the catalytic process. Most of aspartic proteases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin, fungal proteases and processing enzymes such as renin. A second family comprises viral proteases such as the proteases from the HIV also called retropepsin. Metallo proteases are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences but the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of activity (Rao *et al.*, 1998).

2.1.1 Subtilisin-like serine protease

Subtilisin-like serine proteases (EC 3.4.21.62), termed "subtilases" occur in archaea, bacteria, fungi, yeasts and higher eukaryotes. Based on sequence homology, a subdivision into six families is proposed (Siezen and Leunissen, 1997). They are subtilisin family, thermitase, proteinase K, lantibiotic peptidase, kexin and pyrolysin family. Subtilisins are enzymes that are secreted by members of the genus *Bacillus* with subgroups of true subtilisins, high-alkaline proteases and intracellular proteases. The reactions of subtilisin are hydrolysis of proteins with broad specificity for peptide bonds, and showed a preference for a large uncharged residue in